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14. **ABSTRACT**
The retinoblastoma tumor suppressor protein (RB) is functionally inactivated in the majority of human cancers, and nearly half of all breast cancers. RB participates in the growth regulation of breast cancer cells by controlling G1-S phase progression and mediating cell cycle arrest in response to DNA damage and anti-mitogenic signaling. Initially, estrogen-dependent breast tumors are often treated with anti-estrogens, such as tamoxifen or ICI,182,780, while tumors that have become resistant are often treated with DNA-damaging agents, such as ionizing radiation (IR) or cisplatin (CDDP). Although RB loss has been implicated in the bypass of both DNA damaging and anti-estrogenic therapeutic pathways, exploration of the function of RB in breast cancer therapy has been limited. Here, we develop stable clones to recapitulate RB loss in the MCF7 breast cancer model system using siRNA. In this model, we demonstrate that acute RB loss in breast cancer cells results in downstream target deregulation. Additionally, our data reveals that RB loss results in a growth advantage in vitro which is recapitulated in vivo as evidenced by accelerated tumor development in nude mouse xenografts. Interestingly, RB-deficiency in these cells contributes to resistance to hormone ablation therapy in vitro and in vivo. RB knockdown cells are also resistant to DNA damage therapy ultimately leading to increased sensitivity both in vitro and in vivo. Taken together, our data indicate that RB loss in breast cancer facilitates accelerated growth and cellular resistance to two major modes of breast cancer

15. SUBJECT TERMS
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1. Introduction:

Breast cancer is the leading non-cutaneous cancer diagnosis in American women, impacting over 240,000 new patients per year. The retinoblastoma tumor suppressor, RB, is functionally inactivated in virtually every human cancer type and nearly half of all breast cancers [1]. RB participates in the growth regulation of breast cancer cells and it has been demonstrated that its inactivation in mammary tumor models is associated with tumor progression.

RB plays a central role in cell cycle regulation. In quiescent cells, RB is hypophosphorylated and assembles in transcriptional repressor complexes to block cell cycle progression. In response to mitogenic factors, including estrogen in breast cancer, RB is inactivated through hyperphosphorylation catalyzed by the cyclin D-cdk4 and cyclinE-cdk2 complexes [2-4]. These modifications are sufficient to disrupt RB-mediated transcriptional repression and permit cell cycle progression. In contrast, anti-mitogenic factors activate RB, inhibiting cell cycle progression. For example, RB activity is instrumental in the DNA-damage induced cell cycle checkpoint and is necessary for the induction of G_1 and S-phase arrest following DNA damaging events [5]. It is believed that RB elicits this checkpoint by mediating transcriptional repression through interaction with transcription factors such as members of the E2F protein family. Moreover, in breast carcinoma cells RB pathway inactivation is known to occur through many mechanisms including, overexpression of cyclins D1, D3, and E1, and decreased expression or promoter silencing of $p27^{Kip1}$ and $p16^{Ink4A}$, respectively [6]. Each of these mutations occurs at relatively high frequency (30-45% of breast tumors), thus highlighting the importance of RB inactivation in breast cancer [7, 8].

While RB has been shown to be important in carcinogenesis and is a modifier of the anti-mitogenic response, exploration of the function of RB in breast cancer therapy has been limited. Treatment of breast cancer relies on the estrogen dependence of the tumor cells. Two-thirds of all breast cancers are ER-positive, and ER serves as a molecular target for endocrine therapy [9]. Antiestrogens, such as the widely used tamoxifen and the more potent ICI 182780, mediate a G_0/G_1 phase arrest in hormone-dependent cancers [10, 11]. This cell cycle arrest is known to involve a decrease in cyclin D1 gene expression, inactivation of cdk4-cyclin D1 and cdk2-cyclin E complexes, and dephosphorylation of RB [12, 13]. This class of drugs is initially very effective in curbing the growth of ER-positive tumors. Unfortunately however, nearly all of the patients whose tumors initially respond to antiestrogen treatment will develop cellular resistance while maintaining ER-positive disease [14-16]. Because ER is not aberrant in these cancers, this would suggest that cell cycle signaling in response to estrogen may be disrupted. Therefore, it is not surprising that RB functional inactivation and target gene deregulation has been implicated in the bypass of this hormone responsive pathway [17-19]. In addition, high expression levels of known RB target genes, cyclin E and cyclin A, have been demonstrated to be markers of poor response to antiestrogen treatment and survival [20, 21], further highlighting the potential role of RB in breast cancer therapeutic response.

Tumors that exhibit resistance to anti-estrogen therapy have traditionally been treated with DNA-damaging agents such as, ionizing radiation (IR) or chemotherapy including cisplatin (CDDP) [22]. We and others have shown that RB down-regulates specific target genes and elicits cell cycle inhibition in response to DNA-damaging agents [5, 23, 24]. Upon RB functional inactivation, as occurs in tumorigenesis, cells are initially resistant to this these therapies as they able to bypass the G_1/S DNA damage checkpoint despite the presence of deleterious lesions. This has consequence because abrogation of this checkpoint in cells lacking RB can lead to the propagation of mutations, the development of aberrant ploidy, and ultimately apoptosis [25-28]. This would suggest that this line of therapy might ultimately be more efficacious for patients with tumors harboring inactive RB. However, these critical studies have been performed in a variety of primary and tumor-derived cell lines which are not estrogen-dependent and as such are not ideal model systems for the study of breast cancer. Therefore, it is clinically significant to examine the action of RB on cell cycle regulation in response to therapeutic intervention in breast cancer cell lines.

The resistance to conventional therapy is one of the main causes of patient death associated with breast cancer. Analysis of RB function and its effect upon downstream targets in modifying the response to therapeutic agents is imperative for the design of improved treatment strategies. By

recapitulating RB loss in the MCF7 breast cancer cell line, here we interrogate the impact of RB on target gene regulation, cell cycle kinetics, and the response to two distinct lines of therapy: anti-estrogen and DNA damage. We show that RB loss results in modest downstream target deregulation, and this together enables faster growth and bypass of the DNA damage and the anti-estrogen mediated checkpoints. The consequence of the observed checkpoint abrogation is that RB-deficient cells weakly continue to proliferate in the presence of antiestrogens, and display increased sensitivity to DNA damaging agents. Taken together, this work demonstrates that RB is a modifier of the therapeutic response in breast cancer and could be used as a molecular determinant of patient outcome for the two conventional treatment modalities.

2. Body:

Aim 1: Recapitulate RB loss in estrogen-dependent breast cancer cells.

To recapitulate RB loss in the T-47D breast cancer model system, siRNA molecules containing a short hairpin sequence complementary to *Rb* (bp 1261-1279, in the A/B pocket of the protein) or a control lacking the short hairpin sequence (obtained from Dr. Scott Lowe, Cold Spring Harbor Lab) were transfected into cells. Approximately 100 puromycin resistant stable clones were selected and were found to still be expressing the RB protein. Screening for T-47D RB-deficient colonies has been dropped due to poor transfection efficiency and cell death associated with selection. However, the MCF7 RB knockdown clones, as reported last year, have been very successful.

Another method of recapitulating RB inactivation in the MCF7 model system is to overexpress E2F in cells. Although this line of experiments was not originally proposed, it was technically easy to perform in our lab and would bolster our confidence in our RB knockdown data if it was indeed found to be congruent. Specifically, MCF7 cells were infected either with an adenovirus encoding E2F3 (Ad-E2F3) or a control virus (Ad-LacZ) and were harvested 3 days post-infection for immunoblot analysis of levels of known RB-E2F targets (Fig. 1A). Relative to control (Lane 1), the Ad-E2F3 infected MCF7 cells (Lane 2) exhibited significantly increased levels of E2F3, suggesting efficient infection, in addition to increased protein levels of E2F target genes, including PCNA and MCM7. As expected, no changes were detected in RB levels and CDK4 served as a loading control.

Aim 2: Assess the action of RB in the therapeutic response of breast cancer cells to estrogen antagonists and ionizing radiation.

In order to assess the response to therapeutic intervention, 3 days post-infection Ad-E2F3 or Ad-LacZ infected MCF7 cells were separated into 2 major treatment groups; estrogen ablation, or DNA damage therapy. The estrogen ablation group was then subdivided into cells cultured in charcoal dextran treated media (CDT), CDT/Tamoxifen, CDT/ICI 182780, or fetal bovine serum (FBS) as a control for 57 hours prior to labeling with bromodeoxyuridine (BrdU) for an additional 15 hours. Following the 72 total hours of hormone deprivation, cells were harvested and BrdU immunofluorescence performed. The cells in the DNA damage therapy group were similarly used 3 days post adenoviral infection and irradiated with 0 or 5 Gy ionizing radiation. Upon addition of BrdU, the cells were subsequently cultured for 15 hours prior to harvest and BrdU immunofluorescence. For both therapy groups the replicative fraction of treated cells was determined with respect to untreated control cells, which revealed that cells overexpressing E2F3 exhibited significantly reduced levels of cell cycle arrest in each therapeutic condition as compared to the control infected cells (Fig 1B). This data does in fact mimic the RB knockdown data described in the Feb 2004 –Feb 2005 annual report.

The originally described RB-proficient and -knockdown MCF-7 stable clones were used to investigate the impact of RB loss and target deregulation on the cell cycle response to estrogen blockade and DNA-damage *in vitro*. As we have previously assessed the acute cell cycle response to hormone deprivation and DNA damage, we set out to analyze the long term response of cells to these therapies to more closely recapitulate the human condition. Cells were plated at equal densities and either cultured in control media (FBS), CDT/ Tamoxifen (10^{-9}M), CDT/ ICI 182780, or FBS and exposed to 0, 2.5, or 5Gy ionizing radiation (IR). Growth assays in which the cells were counted by trypan blue exclusion every three days (Fig 2A and B) revealed that RB loss increases growth kinetics in the control conditions, FBS or FBS 0 Gy IR. Additionally, RB loss causes resistance to estrogen depletion as cells continued to grow weakly in the tamoxifen and ICI conditions. However, RB loss actually sensitized cells to IR as compared to RB proficient control cells.

Aim 3: Determine the response of breast cancer cell lines with varied RB status xenografted into ovariectomized athymic nude mice to anti-estrogens and ionizing radiation.

While the data described herein provide groundwork from which we can postulate a role of RB in two distinct therapeutic responses, it is critical to perform these studies under more physiologically

relevant conditions. Next we addressed the biological consequence of RB inactivation in breast cancer development and the response to therapy in nude mouse xenografts. Equal numbers of MCF7 donor 1 and si28 cells were injected contralaterally into the flanks of ovariectomized female nude mice supplemented with estrogen to support tumor growth. Control mice lacking the estrogen pellet were also injected, but neither cell type developed into tumors (data not shown). This experiment proved to be an extension of the *in vitro* data as the RB knockdown cells developed into palpable tumors sooner than the controls and continued to grow more quickly as determined by caliper measurement at four day intervals (Fig. 3A). Thirty days post cellular injection, the disparity in size between the RB knockdown tumors and the controls was evident by eye (Fig. 3B). At this time, the mice were injected intraperitoneally with BrdU 1 hour before sacrifice. Upon termination of the experiment, tumors were excised (Fig. 3C) and weighed (Fig. 3D) to confirm that RB knockdown tumors in fact weighed more than the control counterparts. Furthermore, by immunohistochemistry, BrdU incorporation was higher in the RB knockdown tumors as compared to controls, demonstrating increased S-phase entry in RB knockdown tumors (Fig 3E, F).

To assess the response of RB knockdown tumors to therapy *in vivo*, tumors were developed in mice as before and upon reaching a tumor volume of 100-130 mm^3, mice were segregated into three groups; 1. controls (retain estrogen pellet) 2. estrogen depletion (remove estrogen pellet, add tamoxifen pellet) 3. DNA damage (5 mg/kg cisplatin IP every 4 days for 5 courses). Due to unforeseen difficulties with IACUC at the University of Cincinnati, we were unable to proceed with ionizing radiation (IR) treatment as written in the original proposal. Instead, we substituted IR with another DNA damage therapy, the widely used chemotherapeutic drug cisplatin (CDDP). Our results from these experiments show that MCF7 donor tumors respond quickly to tamoxifen by regressing to nearly unmeasurable sizes (Fig. 4A). However, the siRB counterparts did not regress and were able to slowly grow in the tamoxifen group. In the cisplatin arm of the study both tumor types regressed following treatment, however, consistent with the *in vitro* data, the siRB tumors regressed more rapidly throughout the 5 courses of therapy (Fig. 4B). This data, coupled with the *in vitro* BrdU data, would suggest that RB deficient cells are able to continue to cycle in the presence of DNA damage due to a nonfunctional checkpoint, and this eventually is detrimental causing cell death. All tumors were weighed upon excision and, as expected, the tumor weights mirror the growth curve data (Fig. 4C). As in Fig. 3, mice were injected with BrdU prior to sacrifice and tumor sections are currently being stained by immunohistochemistry and counted to determine the percentage of cycling cells in the tumors.

3. Key Accomplishments:

¦ *Task 1*. Recapitulate RB loss in breast cancer model systems.

a. Transfected siRNA molecule into MCF-7 and T-47D cells to inactivate RB in stable clones.
b. Validation of RB loss by immunoblot and immunofluorescence is complete in MCF-7 clones. We were not able to create stable clones in T-47D cell line due to problems with cell viability.
c. Deregulation of RB targets was evident by immunoblot in MCF-7 siRNA clones.

¦ *Task 2.* Elucidate the action of RB in the therapeutic response of estrogen-dependent tumors.

a. RB loss and subsequent target deregulation disrupts estrogen-dependent proliferation pathways, promoting growth in the absence of estrogen or the presence of tamoxifen.
b. RB loss abrogates the DNA damage checkpoint that is evident in control MCF-7 clones and leads to increased sensitivity in RB knockdown clones.

¦ *Task 3.* Determine the response of breast cancer cell lines with varied RB status xenografted into ovariectomized athymic nude mice to anti-estrogens and ionizing radiation.

a. Athymic ovariectomized nude mice were used to implant MCF7 donor and siRB cells into their flanks and assess the role of RB in tumorigenicity. RB- knockdown tumors grew more rapidly than controls.
b. The response of xenograft tumors to estrogen depletion and DNA damage was assessed when tumors reached 100-130 mm^3 via removal of the estrogen pellet and insertion of a tamoxifen pellet, or by treating the mice with 5mg/kg of cisplatin every 4 days for 5 treatments. The tumors in RB knockdown animals were resistant to tamoxifen therapy and more sensitive to DNA damage therapy.

4. Reportable Outcomes:

AWARD:

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University of Cincinnati, Cell Biology Graduate Retreat 1st place (Nov 2005)

PUBLICATIONS:

Bosco EE, Narita M, Aronow BJ, Lowe S, Knudsen ES (In preparation). RB modifies the therapeutic response of breast cancer.

Markey MP, **Bosco EE**, Mayhew CN, Schwemberger SJ, Babcock GF, Jegga AG, Aronow BJ, Reed MF, Knudsen ES. (In Press) Loss of the Retinoblastoma Tumor Suppressor: Differential Action on Transcriptional Programs Related to Cell Cycle Control and Immune Function. *Oncogene*

Gribbon SL., **Bosco EE.**, Parysek LM., Knudsen ES. (In Press) Complex effects of RB loss on Schwann cell biology. *Cancer Letters*

Bosco, EE, Knudsen ES. (2005) Differential Role of RB in Response to UV and IR Damage. *Nuc Acids Res*.

Mayhew CN, **Bosco EE**, Fox SR, Okaya T, Tarapore P, Schwemberger SJ, Babcock GF, Lentsch AB, Fukasawa K, Knudsen ES. (2005) Liver-specific pRB Loss Results in Ectopic Cell Cycle Entry and Aberrant Ploidy. *Cancer Research*

ABSTRACTS/ POSTER PRESENTATIONS:

University of Cincinnati, College of Medicine Poster Forum (Nov 2005)
University of Cincinnati, Cell Biology Graduate Retreat (Nov 2005)
Albert J. Ryan Symposium, Sqwamm Lake, NH (May 2005)

5. Conclusions:

Taken together, our data reveal that loss of RB function in breast cancer facilitates cellular resistance to both hormone ablation and DNA damage therapies. Therefore, these studies uncover a possible mechanism through which breast cancer cells develop therapeutic resistance. Our goal for these findings is to initiate further studies into the value of RB status in breast tumors as a prognostic marker of therapeutic response and ultimately to allow for the implementation of more efficacious therapeutics which will improve patient prognoses.

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7. Appendices:

Figure 1.

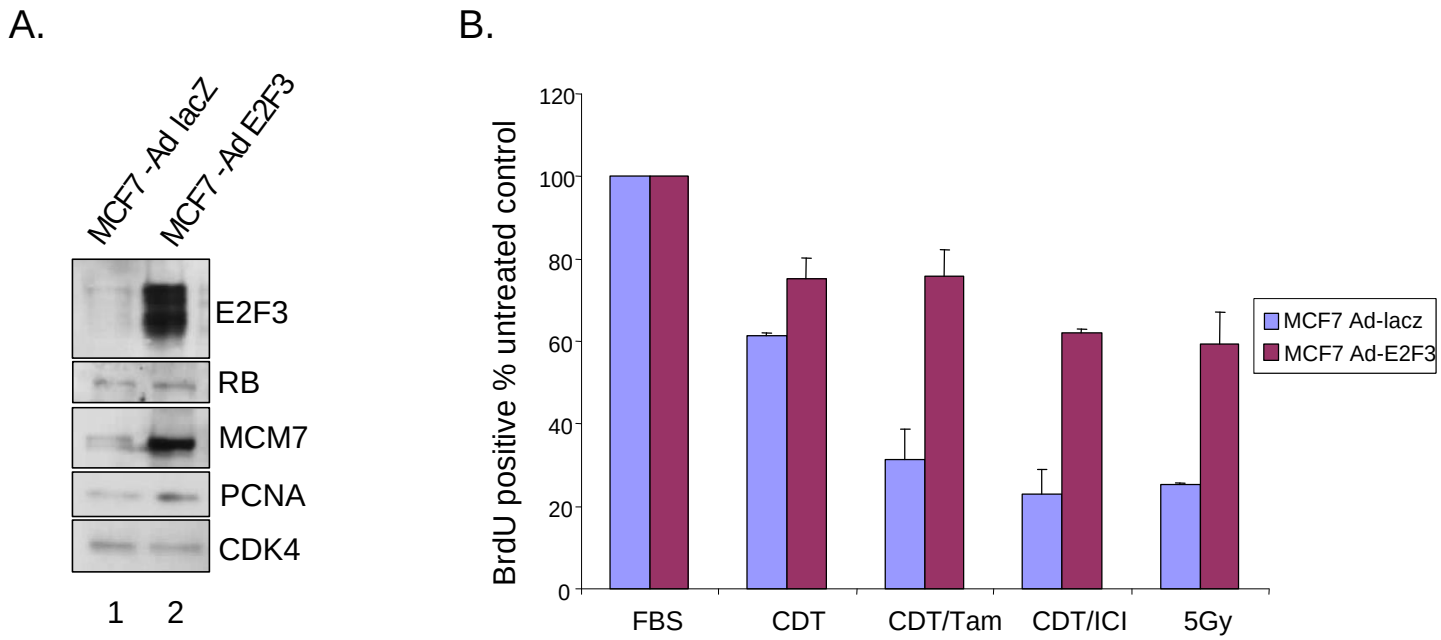
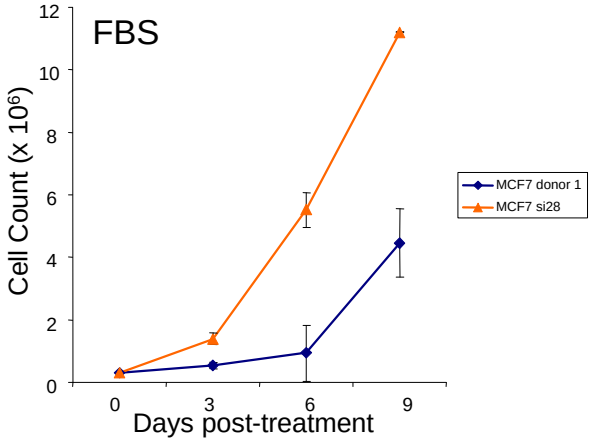
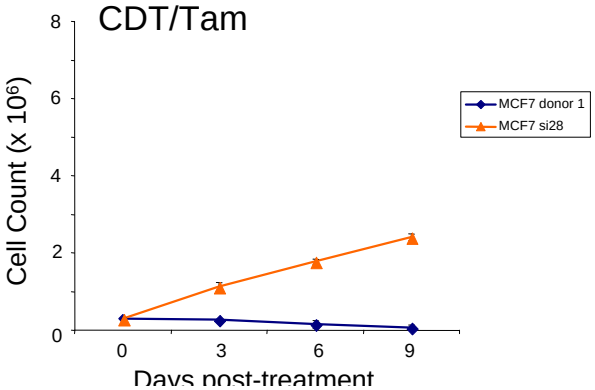


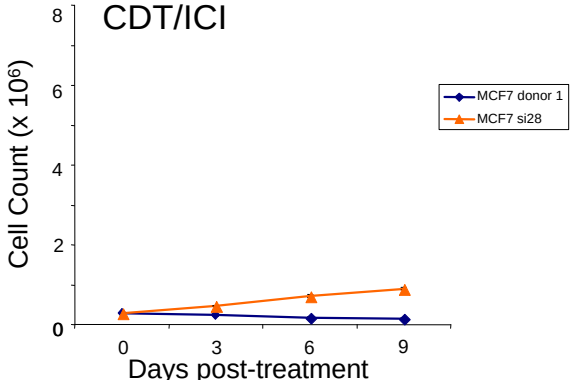
Figure 1. *Recapitualtion of RB loss by E2F3 overexpression in MCF7 cells allows bypass of anti-mitogenic checkpoints*. (A.) MCF-7 cells infected with adenoviral vectors encoding either lacZ or E2F3 were harvested 3 days post-infection, lysed, separated by SDS-PAGE, and immunoblotted for E2F3, RB, MCM7, and PCNA expression levels. Cdk4 served as a loading control. (B.) The adenovirus infected cells from A were cultured in media containing FBS, CDT, CDT/Tam 10^{-9}, or CDT/ ICI 10^{-3} for 3 days or were irradiated with 5 Gy IR prior to BrdU labeling for 18h. Cells were then fixed and BrdU immunofluorescence and scoring was performed.

Figure 2.

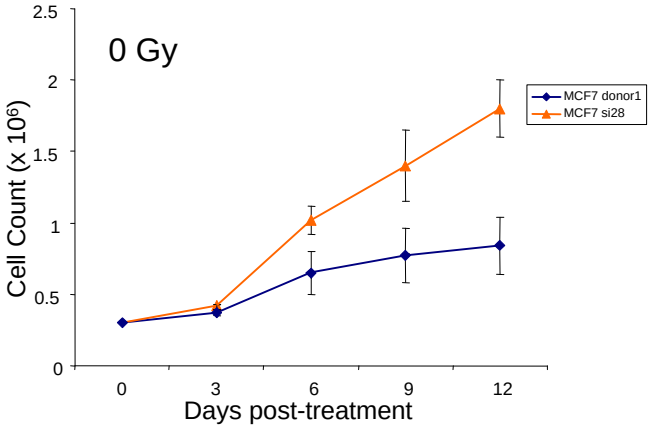
A.

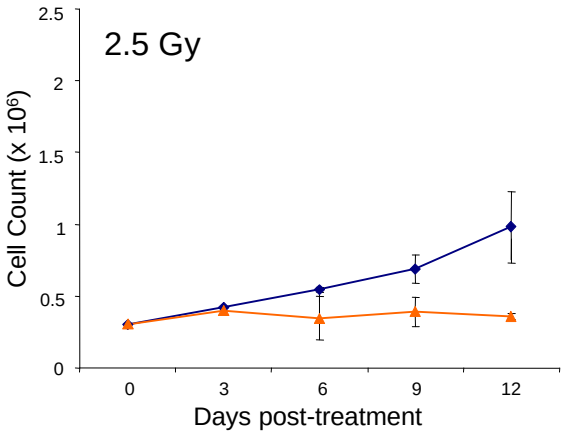

FBS
Cell Count (x 10⁶)
Days post-treatment
MCF7 donor 1
MCF7 si28


CDT/Tam
Cell Count (x 10⁶)
Days post-treatment
MCF7 donor 1
MCF7 si28


CDT/ICI
Cell Count (x 10⁶)
Days post-treatment
MCF7 donor 1
MCF7 si28

B.


0 Gy
Cell Count (x 10⁶)
Days post-treatment
MCF7 donor1
MCF7 si28


2.5 Gy
Cell Count (x 10⁶)
Days post-treatment

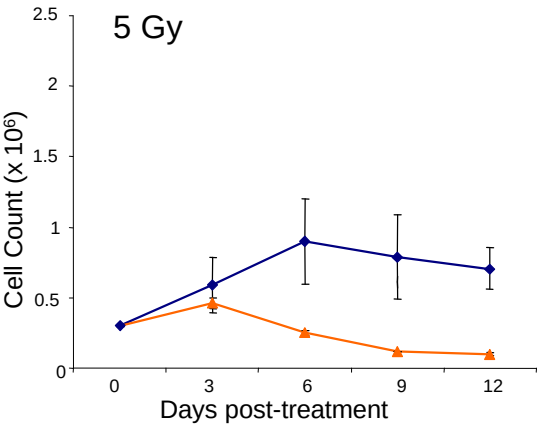
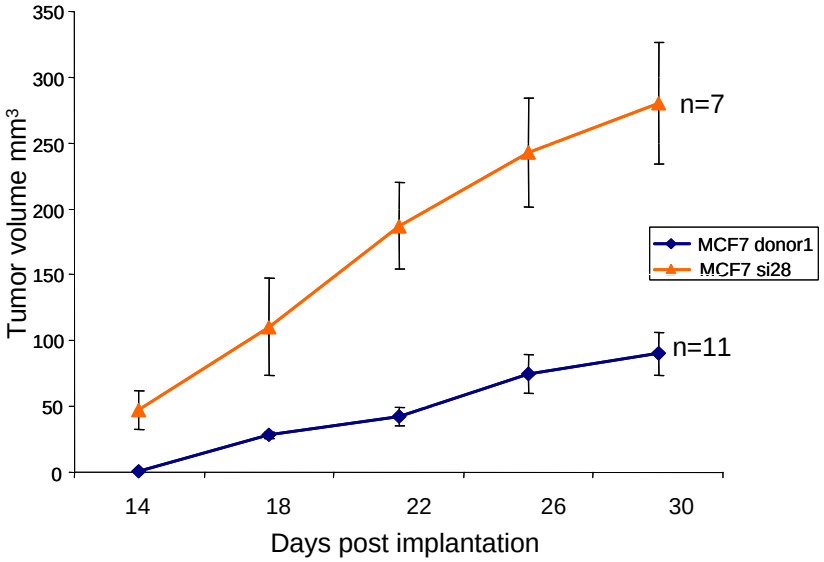

5 Gy
Cell Count (x 10⁶)
Days post-treatment

Figure 2. *RB deficiency promotes resistance to antiestrogen therapy and increased sensitivity to DNA damage therapy.* (A.) MCF-7 donor 1 or si28 cells were seeded at $3x10^5$ and cell growth assays were performed for 9 days while cells were cultured in FBS, CDT/Tam10^{-9}, or CDT/ ICI 10^{-3} and counted every 3 days. (B.) MCF-7 donor 1 or si28 cells were seeded at $3x10^5$, treated with 0, 2.5, or 5 Gy irradiation, and cell growth assays were performed for 12 days while counting every 3 days.

Figure 3.

A.



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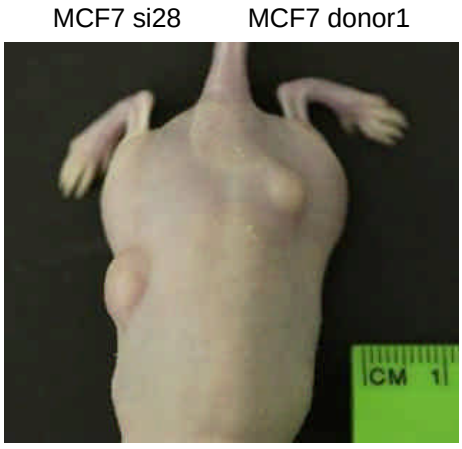
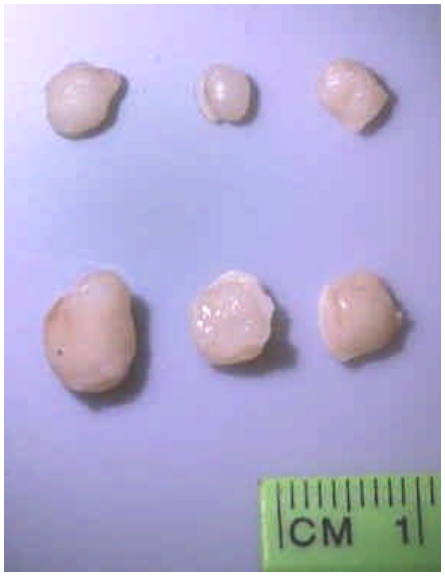


30 days post implantation

C.

30 days post implantation

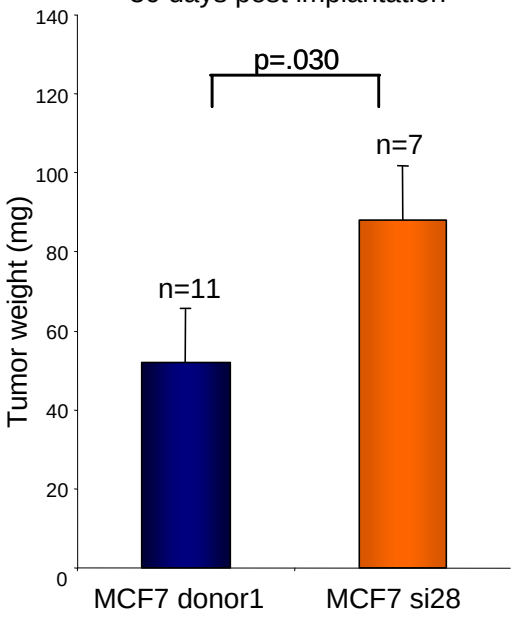


MCF7 donor1

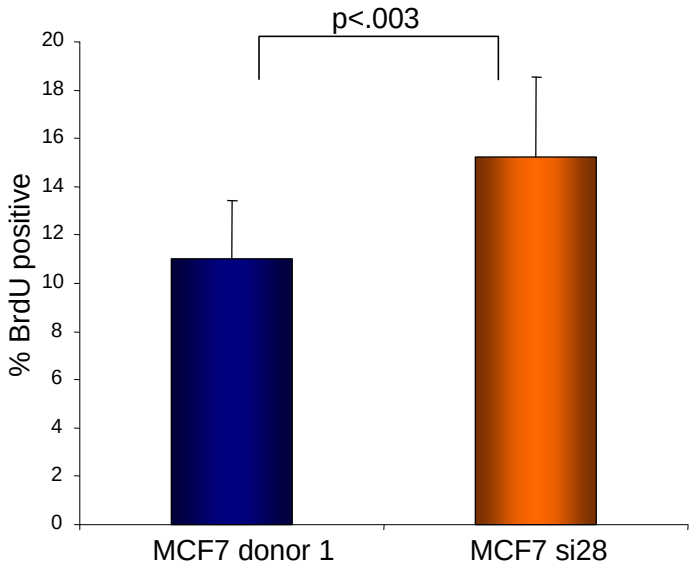
MCF7 si28

D.

30 days post implantation



E.



F.

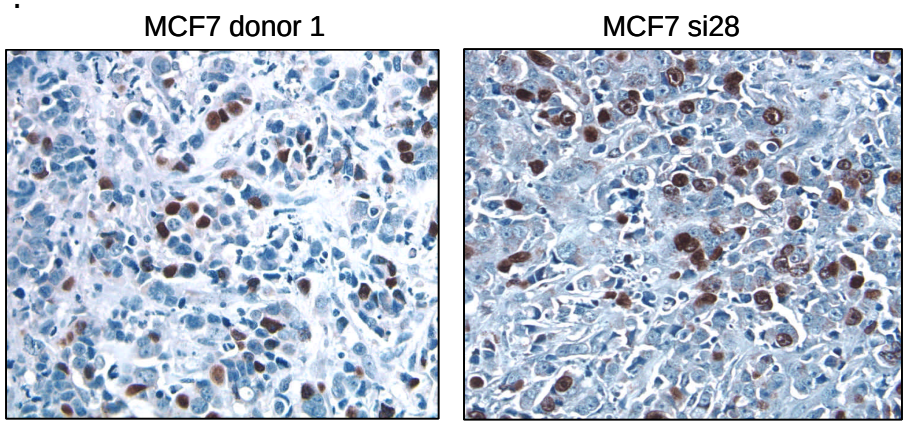
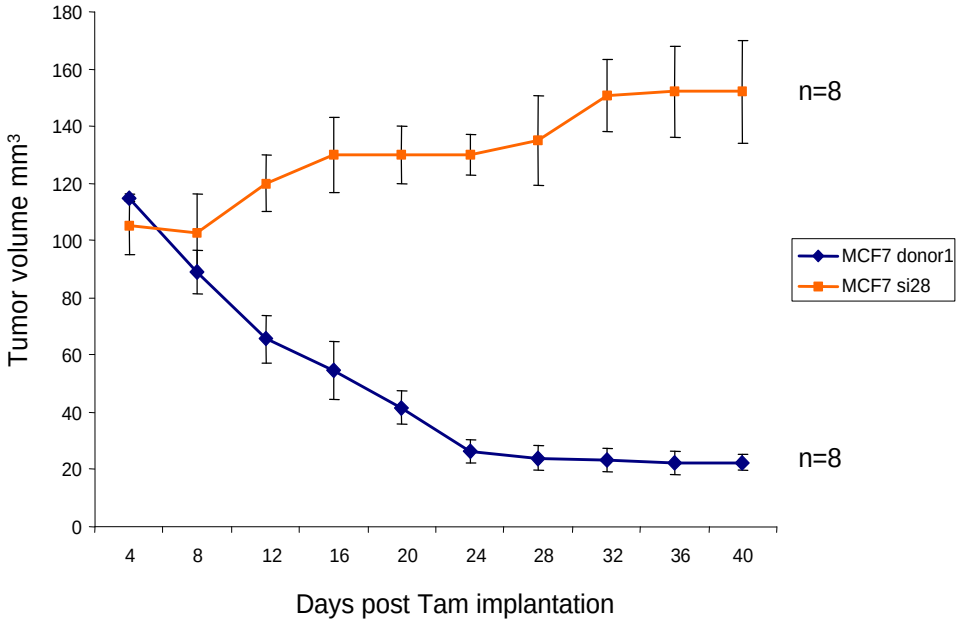


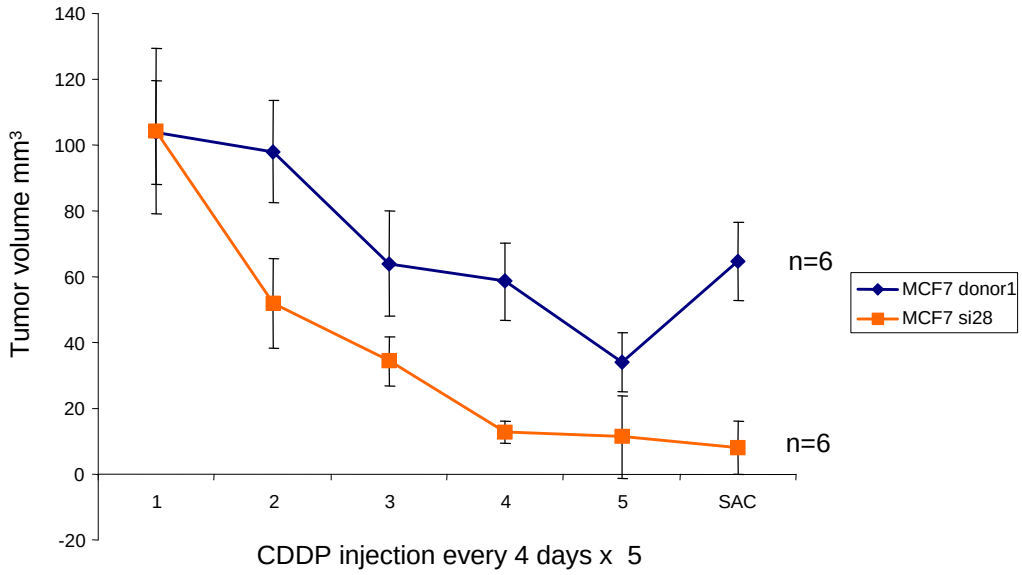
Figure 3. *Tumor growth in nude mouse xenografts is accelerated in RB knockdown cells.* (A.) MCF-7 donor 1 or si28 cells were harvested and resuspended in 3:1 PBS/Matrigel mixture. $2x10^6$ cells in 150 µl of mixture were injected subcutaneously in a contralateral manner in flanks of ovariectomized 5-7 week old nude mice. Mice were supplemented with E2 pellets (1.7mg 90 day release pellet) in the back. Tumors were measured with calipers every 3.5 days and tumor volume was calculated using the equation $V=.52(\text{shorter length})^2(\text{longer length})$. (B.) Example of relative tumor size 30 days post implantation. (C.) 30 days post implantation mice were sacrificed and tumors were excised and weighed. (D.) Tumor weights from C are plotted and a two tailed T-test assuming unequal variances of significance was run to determine p=.030.

Figure 4.

A.


Tumor volume mm3
180
160
140
120
100
80
60
40
20
0
4
8
12
16
20
24
28
32
36
40
Days post Tam implantation
n=8
n=8
MCF7 donor1
MCF7 si28

B.


Tumor volume mm3
140
120
100
80
60
40
20
0
-20
1
2
3
4
5
SAC
CDDP injection every 4 days x 5
n=6
n=6
MCF7 donor1
MCF7 si28

C.

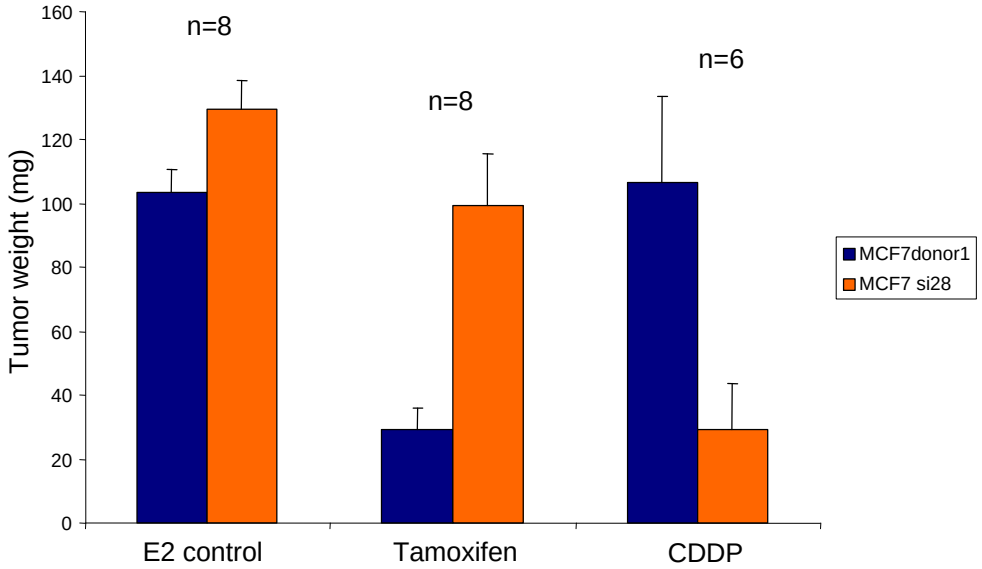

Tumor weight (mg)
160
140
120
100
80
60
40
20
0
n=8
n=8
n=6
E2 control
Tamoxifen
CDDP
MCF7donor1
MCF7 si28

Figure 4. *RB loss enables resistance to hormone ablation therapy and increased sensitivity to DNA damage therapy in xenograft tumors.* Harvested MCF7 donor 1 and si28 cells were resuspended 3:1 in PBS/Matrigel and injected subcutaneously into the left flank of 5-7 week nude mice. Mice were supplemented with E2 pellets as in 5A in order to promote tumor growth. When tumors reached 100-130mm^3, mice were divided into one of three groups, control (retaining the E2 pellet), tamoxifen treated (remove E2 pellet add Tamoxifen pellet), or CDDP treated (retain E2 pellet and inject 5mg/kg CDDP IP every 4 days x 5). (A) Tumor size of the tamoxifen treated animals was monitored by calipers. (B) As in A, growth curves for the cisplatin treated animals. (C) Final tumor weights of all tumors upon excision.